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Abbreviations and definitions:

Acceptable daily intake (ADI)

Antibiotic resistance (AR)

Antimicrobial Resistance (AMR)

Antibiotic-resistance gene (ARG)

Antibiotic-resistant bacteria (ARB) being non-pathogenic environmental bacteria (eARB) or pathogenic (pARB)

Environmental risk assessment (ERA)

Extended-spectrum- β -lactamase (ESBL)

Food and Agriculture Organization (FAO) of the United Nations

Horizontal gene transfer (HGT)

Human health risk assessment (HHRA)

Microbial risk assessment (MRA)

Methicillin-resistant *Staphylococcus aureus* (MRSA)

Multi-criteria decision analysis (MCDA)

Abstract

Background: Only recently has the environment been clearly implicated in the risk of antibiotic resistance to clinical outcome, but to date there have been few documented approaches to formally assess these risks.

Objective: We present possible approaches and identify research needs to enable human health risk assessments (HHRA) that focus on the role of the environment in antibiotic treatment failure caused by antibiotic-resistant pathogens.

Methods: The authors participated in a workshop held in Quebec, Canada, March 4-8, 2012, to define the scope and objectives of how to undertake an environmental assessment of antibiotic resistance risks to human health. We focused on key elements of environmental resistance development ‘hot-spots’, exposure assessment (unrelated to food) and dose-response to characterize risks that may improve antibiotic resistance management options.

Discussion: Various novel aspects to traditional risk assessments were identified to enable an assessment of environmental antibiotic resistance. These include accounting for an added selective pressure on the environmental resistome that over time allows for antibiotic resistant bacterial (ARB) development; identifying and describing rates of horizontal gene transfer (HGT) in the relevant environmental ‘hot-spot’ compartments; and modifying traditional dose-response approaches to address doses of ARB for various health outcomes and pathways.

Conclusions: In this paper we provide a proposal for the inclusion of environmental aspects of antibiotic resistance development in the processes of any HHRA addressing ARB. Due to limited available data a multi-criteria decision analysis (MCDA) approach is suggested as a useful way forward to undertake an HHRA of environmental antibiotic resistance that informs risk managers.

Introduction

This review is based on a workshop held in Québec, Canada in March 2012 which focused on antibiotic resistance in the environment and approaches to assessing and managing effects of anthropogenic activities. The human health concern was identified as environmentally-derived antibiotic-resistant bacteria (ARB) that may adversely affect human health (e.g. reduced efficacy in clinical antibiotic use, more serious or prolonged infection) by either direct exposure of patients to antibiotic-resistant pathogen(s) or by exposure of patients to resistance determinants, and subsequent horizontal gene transfer (HGT) to bacterial pathogen(s) on/within a human host, as conceptualized in Figure 1. Antibiotic-resistant bacterial hazards develop in the environment as a result of direct uptake of antibiotic-resistant genes (ARG) via various mechanisms (e.g. mobile genetic elements such as plasmids, integrons, gene cassettes or transposons) and/or proliferate under environmental selection caused by antibiotics and co-selecting agents such as biocides, toxic metals and nanomaterial stressors (Qiu et al. 2012; Taylor et al. 2011), or by gene mutations (Gillings and Stokes 2012). Dependent on the presence of recipient bacteria these processes generate either environmental antibiotic-resistant bacteria (eARB) or pathogens with antibiotic-resistance (pARB) (Figure 1).

Human health risk assessment (HHRA) is the process to estimate the nature and probability of adverse health effects in humans who may be exposed to hazards in contaminated environmental media, now or in the future (U.S. EPA 2012). In this paper we focus on how to apply HHRA to the risk of infections with antibiotic resistant bacterial pathogens, as they are an increasing cause of morbidity and mortality, particularly so in developing regions (Grundmann et al. 2011). An antimicrobial-resistant microorganism has the ability to multiply or persist in the presence of an increased level of an antimicrobial agent relative to a susceptible counterpart of the same species.

In this paper we limit the resistant group of microorganisms to bacteria and therefore to antibiotic resistance, where the term antibiotic being used as synonymous with antibacterial. It is important to understand the contribution that the environment has on the development of resistance in both humans and animals pathogens, as therapeutic-resistant infections may lead to longer hospitalization, longer treatment time, failure of treatment therapy and the need for treatment with more toxic or costly antibiotics, as well as an increased likelihood of death.

A vast amount of work has been undertaken to understand the contribution and roles played by hospital and community settings in the dissemination and maintenance of antibiotic-resistant bacterial infections in humans. A particular area of focus in terms of exposure in the community setting has been the contribution from the use of antibiotics in livestock production and the presence of eARB and pARB in food of animal origin. In 2011, the Codex Alimentarius released guidelines on processes and methodologies for applying risk analysis methods to foodborne antimicrobial resistance related to the use of antimicrobials in veterinary medicine and agriculture (Codex Alimentarius Commission 2011). The Commission was established in 1963 by FAO and WHO to harmonise international food standards, guidelines and codes of practice to protect the health of consumers and ensure fair trade practices in the food trade.

Furthermore, antibiotics and other antimicrobials are also released into the environment from human sewage (Dolejska et al. 2011), intensive animal husbandry and waste from pharmaceutical manufacture (Larsson et al. 2007). The environmental consequences from the use/release of antibiotics from various sources (Kümmerer 2009a, b) and the HGT of antibiotic-resistance genes between indigenous environmental and pathogenic bacteria and their resistance determinants (Qiu et al. 2012; Börjesson et al. 2009; Cummings et al. 2011; Chen et al. 2011; Chagas et al. 2011; Gao et al. 2012; Forsberg et al. 2012) has yet to be quantified - but is of

global concern (WHO 2012a; Finley et al. 2013). The genetic element(s) encoding for the ability of microorganisms to withstand the effects of an antimicrobial agent are located either chromosomally or extra-chromosomally and may be associated with mobile genetic elements such as plasmids, integrons, gene cassettes or transposons, thereby enabling horizontal and vertical transmission from resistant to previously susceptible strains. From an HHRA point of view, the emergence of ARB in source and drinking waters (De Boeck et al. 2012; Isozumi et al. 2012; Shi et al. 2013) further highlights the need to place these emerging environmental risks in perspective. Yet, assessing the range of environmental contributions to antibiotic resistance may not only be complicated by lack of quantitative data, but also by the need to coordinate efforts across different agencies that may have jurisdiction over environmental risks versus those to human and animal health.

A key consideration for ARB development in the environment is that resistance genes can be present due to natural occurrence (D'Costa et al. 2011). Further, the use of antimicrobials in crops, animals and from human wastes provides a continued entry of antibiotics to the environment, along with possible novel genes and ARB. A summary of the fate, transport and persistence of antibiotics and resistance genes following land application of waste from food animals receiving antibiotics or following outflow to surface water from sewage treatment has emphasized the need to better understand the environmental mechanisms of genetic selection, gene acquisition and dynamics of resistance genes (resistome) and their bacterial hosts (Chee-Sanford et al. 2009; Crtryn 2013). For example, the presence of antibiotic residues in water from pharmaceutical manufacturers in certain parts of the world (Fick et al. 2009), ponds receiving intensive animal wastes (Barkovskii et al. 2012), aquaculture waters (Shah et al. 2012) and sewage outfalls (Dolejska et al. 2011) are considered to be important sources, amongst others,

leading to the presence of ARG in surface waters. In particular, the comparatively high concentrations of antibiotics found in the effluent of pharmaceutical production plants have been associated with an increased presence of ARG in surface waters (Kristiansson et al. 2011; Li et al. 2010; Li et al. 2009). Most recently, 100% sequence identity of ARG from a diverse set of clinical pathogens and common soil bacteria (Forsberg et al. 2012) has highlighted the potential for environmental HGT between eARB and pARB.

Despite these concerns, there are few risk assessments that evaluate the combined impacts of antibiotics, ARG and ARB in the environment on human and animal health (Keen and Montforts 2012). Recent epidemiological data have included assaying for ARB in drinking water and the susceptibility of commensal *Escherichia coli* in household members. Water was a risk factor but so were other factors not directly related to the local environment that accounted for the presence of resistant *E. coli* in humans (Coleman et al. 2012). In many studies, native bacterial members of drinking water systems are clearly able to accumulate ARG (Vaz-Moreira et al. 2011).

In addition to addressing environmental risks arising from the development of antibiotic resistance, there is also the low probability but high impact ‘one-time-event’ type of risk event, to consider. This exceedingly rare event that results in the transfer of a novel (to clinically important bacteria) resistance gene from a harmless, environmental bacterium to a pathogen, need only happen once should a human be the recipient of the novel pARB. Unlike the emergence of SARS and similar viruses where, in hindsight, the risk factors are now well understood (Swift et al. 2007), the conditions for a ‘one-time-event’ could occur in a range of ‘normal’ habitats. Once developed, the resistant bacterium/gene has a possibility to spread between humans around the world (such as seen with the spread of New Delhi metallo-beta-lactamase-1 (NDM-1) resistance (Wilson and Chen 2012)), promoted by our use of antibiotics.

While it appears very difficult to quantify the probability for such a rare event (including assessing the probability for where it will happen and when), there is considerable value in trying to identify the risk factors (such as pointing out critical environments for HGT to occur, identifying pharmaceutical exposure levels that could cause selection pressures and hence increase the abundance of a given gene). After such a critical HGT event, then we may move into a more quantitative kind of HHRA.

The overall goal of the workshop was to identify the significance of ARB within the environment and to map out some of the complexities involved, so as to identify research gaps and provide statements on the level of scientific understanding for various ARB issues. As such a broad range of international delegates spanning academics, government regulators, industry members and clinicians discussed various issues. The focus of this paper came from discussions to improve our understanding of the human health risks, in addition to epidemiological studies, given the need to develop human health risk assessment approaches to explore potential risks and inform risk management. As the end goal of an assessment depends on the context (research, regulation etc.), this paper provides a generic approach to undertake a human health risk assessment of environmental ARB that can be adapted to the users' interest (conceptualized in Figure 1). Given the many uncertainties, identified research gaps are also highlighted.

General considerations for an assessment of environmental ARB risks

Understanding other on-going relevant international activities and the types of antibiotics used provide good starting points to aid in framing a risk assessment of ARB. A report from an Ad-Hoc Task Force of the Codex Alimentarius Commission (2011) describes eight principles that are specific to foodborne antimicrobial resistance risk analysis, several of which are generally

applicable to a human health risk assessment of environmental ARB as bulleted at the end of this section. Examples include the recommendations of the “Joint FAO/WHO/OIE Expert Meeting on Critically Important Antimicrobials” (FAO/WHO/OIE 2008) and the “WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (WHO-AGISAR)” (WHO 2012b), which provide information for setting the priority antibiotics for a human risk assessment. It should also be noted that there are significant national and regional differences in the use of antibiotics, resistance patterns, and human exposure pathways.

In general, risk assessments are framed by identifying risks and management goals, so that the assessment informs the need for possible management options and enables evaluation of management success. There was consensus at the workshop that management could best be applied at points of antibiotic manufacturing and use, agricultural operations including aquaculture, and wastewater treatment plants (Pruden et al. 2013). Nonetheless, assessing the relative impact of managing any particular part of a system is hampered by the lack of knowledge on their relative importance for the overall risk. That is, as the WHO stated recently (2012a): “*the emergence of AMR is a complex problem driven by many interconnected factors; single, isolated interventions have little impact*”. Hence, a starting point for an assessment of environmental antibiotic-resistance risks intended to aid risk management is a ‘theoretical risk assessment pathway’ based on local surveillance data on the occurrence and types of antibiotics used in human medicine, crop production, animal husbandry and companion animals, information on ARG and ARB in the various environmental compartments (in particular soil and aquatic systems including drinking water) and related disease information. This should be amended by discussion with the relevant stakeholders, which requires extensive risk communication and could form part of the multi-criteria decision analysis (MCDA) approach

discussed in detail in the following sections. Pruden et al. (2013) also advocate coupling environmental management and mitigation plans with targeted surveillance and monitoring efforts, in order to judge the relative impact and success of the interventions.

Examining Figure 1 it becomes clear that some details require quantitative measures to undertake a useful human health risk assessment. Hence the key issue is how estimates can be made by experimental and modeling approaches. Furthermore, there are hazard concentration, time and environmental compartment-dependent aspects. Firstly, for environmental bacteria (including pathogens that may actively grow outside of hosts) to develop into eARB/pARB (processes 1 and 2, Figure 1), the current understanding is that for non-mutation derived antibiotic-resistance a selective pressure (i.e. presence of antibiotics or antibiotic-resistance determinants) must be maintained over some time in the presence of antibiotic determinants; for existing pARB released into the environment, survival in environmental media is the critical factor. However, the exact mechanisms and quantitative relationships between selective pressures and ARB development have yet to be elucidated, and may be different depending upon the antibiotic, the bacterial species and resistance mechanisms involved. In cases where selective pressure is removed, the abundance of antibiotic-resistance ARB may be reduced, but not to extinction. (Cottell et al. 2012; Andersson and Hughes 2010, 2011). Even a minority of ARB at the community level represents a reservoir of ARG for horizontal transfer once pressure is reapplied. Overall, as it seems inevitable that ARB will eventually develop against any antibiotic (Levy and Marshall 2004), the key management aim would seem to be to delay and confine such a development as much as possible.

Secondly, a robust quantitative risk assessment will require rates of HGT and/or gene mutations in the relevant compartments (processes 3-5 in Figure 1) to be described for different

combinations of donating eARB and receiving pARB strains. The lack of quantitative estimates for mutation/HGT of ARG is a major data gap.

Thirdly, traditional microbial risk assessment dose-response approaches (captured in processes 6 and 8 of Figure 1) could be used to address the likelihood of infection (Codex Alimentarius Commission 2011; U.S. Department of Agriculture/Food Safety and Inspection Service (USDA/FSIS) and U.S. Environmental Protection Agency (EPA) 2012), but the novel aspect required here, in addition to HGT and ARB selection, would be to address quantitative dose-response relationships for eARB (in the presence of a sensitive pathogen in/on a human), (Processes 3 and 6, Figure 1). Importantly, the key difference from traditional HHRA undertaken in some jurisdictions is that it is essential to include environmental processes to fully assess human risks associated with antibiotic resistance.

Therefore, the sort of information that should be documented for a human health oriented risk assessment of environmental ARB includes [adapted from (Codex Alimentarius Commission 2011)]:

- Clinical and environmental surveillance programmes for antibiotics, ARB and their determinants, with a focus on regional data reporting the types and use of antibiotics in human medicine, crops and for commercial and companion animals, and globally for where crop and food animals are produced;
- Epidemiological investigations of outbreaks and sporadic cases associated with ARB, including clinical studies on the occurrence, frequency and severity of ARB infections;
- Identification of the selection pressures (time and dose of selecting/co-selecting agents) required to select for resistance in different environments, and subsequent HGT to

human-relevant bacteria – based on a summary of previously identified ‘hot-spots’ describing the frequency of HGT and uptake of ARG into environmental bacteria, including environmental pathogens;

- Human, laboratory and/or field animal/crop trials addressing the link between antibiotic usage and resistance (particularly regional data);
- Investigations of the characteristics of ARB and their determinants (ex-situ and in-situ);
- Studies on the link between resistance, virulence and / or ecological fitness (e.g. survivability or adaptability) of ARB;
- Studies on the environmental fate of antibiotic residues in water/soil and their bioavailability associated with selection of ARB in any given environmental compartment, animal or human host resulting in pARB; and
- Existing ARB and related pathogen risk assessments.

In summary, there are multiple data sources required to undertake a human health risk assessment for environmental ARB, where much of the data may be severely limited (particularly for a quantitative assessment). Hence the final risk assessment report has to put particular emphasis on discussing the evidence trail and weight of evidence for each finding. Furthermore, when models are constructed, previously unused datasets should be considered for model verifications where possible.

Applicability of traditional Risk Assessment approaches

Human health risk assessment of antibiotics in the environment builds from traditional chemical risk assessments (National Research Council 1983), starting for example, with an acceptable daily intake (ADI) based on resistance data (VICH Steering Committee 2012). A corresponding

metric for environmental antibiotic concentration could be developed based on the concept of the minimum selective concentration (MSC) (Gullberg et al. 2011), defined as the minimum concentration of an antibiotic agent that selects for resistance. A major difference from the traditional chemical risk assessment approach is that, as with the MSC assay, there is also a need to address the human health effects arising from ARGs and resistance determinants that give rise to ARB, including resistance associated with mutations (processes 1 and 2, Figure 1). In the absence of specific data, a MSC assay could inform a risk assessor on the selective concentration of a pharmaceutical or complex mixture of compounds in a matrix of choice, to begin to describe thresholds for significant ARB development.

Moving on from antibiotic to pathogen risks, these may be evaluated through Microbial Risk Assessment (MRA), a structured, systematic, science-based approach that builds on the chemical risk assessment paradigm, involving problem formulation (describing the hazards, risk setting and pathways), exposure assessment of the hazard (ARB), dose-response assessment that quantifies the relationship between hazard dose and pARB infection in humans (processes 6 and 7, Figure 1), and their combination to characterize risk for the various pathways of exposure to pathogens identified to be assessed. MRA is used qualitatively or quantitatively to evaluate the level of exposure and subsequent risk to human health from microbiological hazards. In the context of antibiotic-resistant microorganisms, environmental MRA is in its infancy, but needs to address resistant bacteria and/or their determinants. MRA was originally developed for faecal pathogen hazards in food and water (ILSI 1996), with more recent modifications to include biofilm-associated environmental pathogens, such as *Legionella pneumophila* (Schoen and Ashbolt 2011). Some human pathogens can grow in the environment (and may become pARB,

processes 1 and 2, Figure 1), and many will infect only compromised individuals, hence they are generally referred to as opportunistic pathogens.

Over the past 20 years, MRA has largely evolved by input from the international food safety community, and it is now a well-recognized and accepted approach within food safety risk analysis. In 1999, Codex Alimentarius adopted the “Principles and Guidelines for the Conduct of Microbiological Risk Assessment CAC/GL-30” (Codex Alimentarius Commission 2009). More recently Codex published guidelines for risk analysis of foodborne antimicrobial resistance (Codex Alimentarius Commission 2011), and in the US, MRA guidelines for food and water for federal US agencies (U.S. EPA and USDA, 2012), which continue to use the four-step framework originally described for chemical risk assessment. Several ARB risk assessments have been published and reviewed in recent years (McEwen 2012; Geenen et al. 2010; Snary et al. 2004). However, nearly all of these studies focus on foodborne transmission; human health risk assessments dealing with ARB transmission via various environmental routes or direct contact with ARG are sparse.

For example, Geenen et al. (2010), studied extended-spectrum beta-lactamase [ESBL]-producing bacteria and identified the following risk factors: previous admission to health-care facilities, antimicrobial drugs usage, travelling to high-endemic countries and the presence of ESBL-positive family members. They concluded by stating that an environmental risk assessment (ERA) would be helpful in addressing the problem of ESBL-producing bacteria, but noted that none had been performed.

Hazard Identification and Hazard Characterization

Unfortunately, the authors are unaware of data that quantitatively link ARG uptake and human health effects (processes 3 and 6, Figure 1). What does exist in general and is rapidly improving in quality, however, are data on the presence of ARGs within various environmental compartments (Cummings et al. 2011; Allen et al. 2009; Ham et al. 2012), and specifically of clinically relevant resistance genes within soils (Forsberg et al. 2012) (process 1, Figure 1). As described previously, precursors that lead to the development of antibiotic-resistant bacterial hazards include ARG and mechanisms to mobilise these genes, antibiotics and co-selecting agents (Qiu et al. 2012; Taylor et al. 2011) along with gene mutations (Gillings and Stokes 2012). Dependent on the presence of recipient bacteria these processes generate eARB or pARB (processes 1 and 2, Figure 1).

While the pathways for human exposure to ARB hazards are further described in the Environmental Exposure Section, the focus here is on the development and characterization of antibiotic-resistant bacterial hazards. The authors are not aware of comprehensive data being available for the numerous parameters relevant to individual environmental compartments that describe: 1) antibiotic resistance development by antibiotics and other co-selecting agents, 2) the flow of antibiotic-resistance genes (resistome) and acquisition elements (e.g. integrons) in native environmental compartment bacteria, and 3) the likely range in rates of horizontal and vertical gene transfer within environmental compartments. Nonetheless, factors considered important include the range of potential pathways involving the release of antibiotics, ARG and ARB into and amplifying in environmental compartments such as the rhizosphere, bulk soil, compost, biofilms, wastewater lagoons, rivers, sediments, aquaculture, plants, birds, and wildlife.

With respect to antibiotics, in general what is required to aid hazard characterization is a listing of the local antibiotic classes of concern, what is known of their environmental fate and where they may accumulate in particular environmental compartments (such as the rhizosphere versus general soil, compost, biofilms, wastewater lagoons, rivers, sediments, aquaculture, plants, birds, wildlife, farm animals and companion animals). Selection for ARB (process 2, Figure 1) will depend on the type and in-situ bioavailability of selecting/co-selecting agents, abundance of bacterial host, as well as the abundance of AR determinants.

Selection for ARB is further modulated by the nutritional status of the relevant bacterial community members as high metabolic activity and high cell density promote bacterial community succession and HGT (Sørensen et al. 2005; Brandt et al. 2009). By contrast, HGT is relatively independent of the antibiotic, although antibiotics and ARB may be co-transported (Chen et al. 2013), and increases in HGT rates are thought to occur in stressed bacteria. For example integrase expression can be up-regulated (increased) by bacterial SOS response in the presence of certain antibiotics (Guerin et al. 2009).

While quantitative data that describe the development of pARB in the environment are missing, ample evidence exists for the co-uptake by an antibiotic-sensitive pathogen in the presence of antibiotic, ARG (such as on a plasmid with metal resistance and/or carbon utilization genes (Laverde Gomez et al. 2011; Knapp et al. 2011), or as demonstrated in-vitro for a disinfectant/nanomaterial (Qiu et al. 2012; Soumet et al. 2012).

The spatial distribution of organisms will also impact on transfer (opportunity for close proximity), which result from inherent patchiness, soil structure, presence of substrates and so forth. In considering gene transfer rates, there may be 'hot-spots' of activity, for example, there

is evidence for HGT of clinically-relevant resistance genes between bacteria in manure-impacted soils (Forsberg et al. 2012), and in association with the rhizosphere due to organic-rich conditions (Pontiroli et al. 2009). Likewise, selection pressures for subsequent proliferation of eARB may be higher in these ‘hot-spot’ environments (Brandt et al. 2009; Li et al. 2013). Therefore, it is important to recognize likely zones of high activity during the problem formulation and hazard characterization stages of a risk assessment, and when using sampling to identify in-situ exchange rates. As an example marker of anthropogenic impact with potential to predict the impact on the mobile resistome, class 1 integrons could be used, given their ability to integrate gene cassettes that confer a wide range of antibiotic and biocide resistance (Gaze et al. 2011). In semi-pristine soils, prevalence may be two or three orders of magnitude lower than in impacted soils and sediments (0.001 vs. 1% respectively) (Gaze et al. 2011; Zhu et al. 2013).

In addition to a huge diversity of eARB hazards, reference pathogens used in microbial risk assessments, which may acquire ARG as illustrated in Figure 1, include: (1) foodborne and waterborne faecal pathogens represented by *Campylobacter jejuni*, *Salmonella enterica* or various pathogenic *E. coli*, and (2) environmental pathogens, such as respiratory, skin or wound pathogens represented by *Legionella pneumophila*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Each of these faecal and environmental pathogens are well known to be able to acquire ARG, hence given further data on environmental HGT rates, they could make good examples of what are referred to as reference pathogens. However, what is much more problematic for a risk assessment, and a current limiting factor, is to describe the rate at which the indigenous bacteria transfer resistance to these pathogens within each environmental compartment and the human/animal host (processes 3-5, Figure 1). Methods to model and experimentally derive relevant information on these environmental issues are discussed within

the Environmental Exposure Assessment section below. Data on HGT within the human gastrointestinal tract have been summarised by (Hunter et al. 2008).

Dose-Response relationships

To properly characterize human risks it is typical to select hazards for which there are dose-response health data described either deterministically or stochastically, as available for the reference enteric pathogens identified above (Schoen and Ashbolt 2010), but have yet to be quantified for the skin/wound reference pathogens (Mena and Gerba 2009; Rose and Haas 1999). However, as noted above (addressing processes 1-5, Figure 1), an important difference for ARB is the need to account for the phenomena associated with selective environmental pressures for the development of ARB, then HGT, ultimately to form the human infective dose of either eARB or pARB. The exact mechanisms and dose-response relationships have yet to be elucidated, and may be different depending on the bacterial species and resistance mechanisms involved. Nonetheless, it seems reasonable for the non-compromised human exposed to a pARB to fit the published dose-response infection relationship (e.g. derived from ‘feeding’ trials with healthy adults or from information collected during outbreak investigations) for strains of the same pathogen without antibiotic-resistance. What appears more limiting are dose-response models describing the probability of illness based on the conditional probability of infection, and for people already compromised, such as those undergoing antibiotic therapy. However, while there is definitive data on pARB being more pathogenic or causing more severe illness than their antimicrobial-susceptible equivalents (Helms et al. 2004; Helms et al. 2005; Barza 2002; Travers and Barza 2002), that may not always be the case (Evans et al. 2009; Wassenaar et al. 2007). Nonetheless, clear examples of excess mortality include associated blood stream infections (BSIs) for methicillin resistant *Staphylococcus aureus* (MRSA) and from 3rd generation

cephalosporin resistant *E. coli* (G3CREC). In 2007, 27,711 cases of MRSA were associated with 5,503 excess deaths and 255,683 excess hospital days in participating European countries and 15,183 episodes of G3CREC BSIs caused 2,712 excess deaths and 120,065 extra hospital days (de Kraker et al. 2011). The authors predict that the combined burden of resistance of MRSA and G3CREC will likely lead to a predicted incidence of 3.3 associated deaths per 100,000 inhabitants in 2015. Yet for many regions of the world, such predictions are less well understood.

The final step of MRA is risk characterization, which integrates the outputs from the hazard identification, the hazard characterization, dose-response and the exposure assessment (discussed in the next section) with the intent to generate an overall estimate of the risk. This estimate may be expressed in various measures of risk, for example in terms of individual or population risk, or an estimate of annual risk based on exposure to specific hazard(s). Depending on the purpose of the risk assessment, the risk characterization can also include the key scientific assumptions used in the risk assessment, sources of variability and uncertainty, and a scientific evaluation of risk management options.

Environmental exposure assessment

Based on our conceptualization of the processes important to undertake HHRA of ARB (Figure 1), most elements related to ARB development in environmental media (processes 1, 2 and 4) have been addressed in the Hazard Identification and Hazard Characterization section. Here we focus on describing important environmental compartments for and human exposure to ARB (processes 3 and 6, Figure 1). Hence, critical to exposure assessment are the concentrations of selecting environmental factors (such as antibiotics) and along with ARB, their fate and transport to points of human uptake. For a particular human health risk assessment of ARB it would be

important to select/expand on individual pathway scenarios (identifying critical environmental compartments to human contact) relevant to the antibiotic/resistance determinants identified in the problem formulation and hazard characterization stages.

Compartments of potential concern include soil environments receiving animal manure or biosolids, compost, and lagoons, rivers and their sediments receiving wastewaters (Chen et al. 2013). More traditional routes of human exposures to contaminants that could include eARB and pARB are drinking water, recreational and irrigation waters impacted by sewage and antibiotic production wastewaters, food, exposure to air impacted by farm buildings and exposure to farm animal manures as discussed by Pruden *et al.* (2013). What is emerging as an important research gap is the in-situ development of ARB within biofilms (Boehm et al. 2009) and their associated free-living protozoa that may protect and transport ARB (Abraham 2010) to and within drinking water systems (Schwartz et al. 2003; Silva et al. 2008). This latter route could be particularly problematic for hospital drinking water systems where an already vulnerable population is exposed. Also with the increasing use and exposures to domestically-collected rainwater, atmospheric fall-out of ARB may ‘seed’ household systems (Kaushik et al. 2012).

Having identified antibiotic concentrations and pathogen densities in the environment, and possible levels/rates of ARB generation in each environmental compartment, a range of fate and transport models are available to estimate the amounts of antibiotics, pathogens, ARB and ARG at points of human contact (processes 3 and 6, Figure 1). Such models are largely based on hydrodynamics, with pathogen-specific parameters to account for likely inactivation/predation in soil and aquatic environments, such as sunlight inactivation (Cho et al. 2012; Ferguson et al. 2010; Bradford et al. 2013). A key aspect of the fate and transport models is to account for the inherent variability of any system component. In addition, our uncertainties in assessing model

parameter values should also be factored into fate and transport models such as by using Bayesian synthesis methods (Williams et al. 2011; Albert et al. 2008). More recent models are including Bayesian learning algorithms that help to integrate information using meteorologic, hydrologic, and microbial explanatory variables to better account for parameter uncertainties (Motamarri and Boccelli 2012; Dotto et al. 2012). Overall, these models also help to identify management opportunities to mitigate exposures to ARB/antibiotics and are an important aspect to include in describing the pathways of hazards to points of human exposure in any risk assessment.

Multi-criteria decision analysis (MCDA) and risk ranking methods

Considering the complexity of exposure pathways associated with environmental ARB risks and the large uncertainty in the input data for some nodes along the various exposure pathways, outputs would inevitably be difficult to interpret by decision-makers, and could in fact be counter-productive. As such, there is merit in considering decision analysis approaches for prioritizing risks, to guide resource allocation and data collection activities, and to facilitate decision-making. Whereas there is a range of ranking options, use of weightings, selecting criteria (Pires and Hald 2010; Cooper et al. 2008) and failure mode and effects analysis (Pillay and Wang 2003); overall in the area of microbial risk assessment there is a consolidation to MCDA approaches, that may include Bayesian algorithms (Ruzante et al. 2010; Lienert et al. 2011; Ludwig et al. 2013). Hence, further discussion of MCDA follows next.

Approaches such as MCDA are designed to provide a structured framework for making choices where multiple factors need to be considered in the decision-making process. MCDA is a well-established tool that can be used for evaluating and documenting the importance assigned to

different factors in ranking risks (Lienert et al. 2011), albeit heavily dependent on expert opinion. In the context of MRA it has been used to rank foodborne microbial risks based on multiple factors including public health, market impacts, consumer perception and acceptance, and social sensitivity (Ruzante et al. 2010), as well as to prioritize and select interventions to reduce pathogen exposures (Fazil et al. 2008). Examples of MCDA applications in structuring decisions for managing ecotoxicological risks have also been reported (Linkov et al. 2006; Semenzin et al. 2008) and provide useful MCDA approaches to consider. MCDA could be used, for example, to evaluate and rank the relative risks between habitats highly polluted with antibiotics, ARG and their determinants, as described above as possible ‘hot-spots’ for HGT and development of ARB. Likewise, it could be applied to evaluate the relative contribution of co-selecting agents (e.g. detergents, biocides, metals, nanomaterials) from various sources to the overall risk of ARB in the environment. Moreover, for a range of antibiotics considered to be of environmental concern, MCDA approaches could be used for risk ranking according to criteria based on relevant contributing factors, which have been discussed here (e.g. mobility of resistance determinants in genetic elements, antibiotic resistance transfer rates in different environmental compartments, accumulation levels of antibiotics in environmental compartments, environmental fate and transport to exposure points). It is also important to identify in the MCDA process the low probability but high impact ‘one-time-event’ types of risk event, as raised in the introduction.

Because MCDA techniques rely on expert opinion (which is often regarded as a limitation of such approaches), well-structured and recognized elicitation practices should be used in order to avoid introduction of biases and errors by subjective scoring. On the flipside, one of their main advantages is that they capture a consensus opinion among an expert group about the most relevant criteria and their relative weight on the decision.

Important research gaps to progress HHRA of antibiotic resistance

The importance of environmental selection for ARB was identified in the workshop, as were areas discussed here as research gaps. In particular, specific attention should be paid to contaminated habitats ('hot-spots') in which antibiotics, co-selecting agents, bacteria carrying resistance determinants on mobile genetic elements and favourable conditions for bacterial growth and activity prevail at the same time – all conditions expected to favour HGT. However, as such data are currently very limited, alternative ways and possible experimental methods to address these data gaps for human health risk assessment were also evaluated during the workshop, as now summarized along the process steps identified in Figure 1.

Assays to determine minimum selective concentration (MSC) – Processes 1, 2 and 4

Assays could be developed to measure minimum selective concentration (MSC) (Gullberg et al. 2011) for a range of antibiotics and environmental conditions. For example, assays could be developed and validated in sandy and clay soils, different sediments, and water types, with isogenic pairs of the model organism inoculated into the matrix of choice and subjected to a titration of the selective agent to sufficiently high dilution. Selection at sub-inhibitory concentrations and assay development for environmental matrices is a key area of research that needs addressing, but overall care is needed when interpreting ex-situ studies and extrapolating to in-situ environmental conditions, including how to deal with ill-defined hazard mixtures in the environment.

Assays to identify environmental 'hot-spots' – for Processes 1, 2 and 4

Here we define 'hot-spots' as locations where a high-level of HGT and antibiotic-resistance develop. Hot-spots may for instance include aquatic environments affected by pharmaceutical

industry effluents, aquaculture or sewage discharges and terrestrial environments affected by the deposition of biosolids or animal manures. The degree of persistence of antibiotic resistance (i.e. the rate by which resistance disappears without an environmental selection pressure for resistance) must also be considered for risk assessment and will depend on the fitness cost of resistance. However, the fitness cost within complex and variable environments are not easy to assess. Furthermore, standard methods for evaluating environmental selection pressures in complex microbial communities are not developed, but several experimental approaches are possible and are described elsewhere (Brandt et al. 2009; Berg et al. 2010).

The approaches identified could be lab based (to assess the potency of known compounds/mixtures) or applied in the field to assess if the environment in question (with its unknown mixture of chemicals present etc.) is a ‘hot-spot’. Defining “critical exposure levels” is therefore an important HHRA output to aid management activities, which will likely vary between environmental compartments and within, depending on the location.

Screening for novel resistance determinants – to reduce Process 2

Screening procedures could be introduced early in the development cycle of novel antibiotics to ensure that existing resistance determinants are not prevalent in environmental compartments. Marked recipient strains could be inoculated into environmental matrices e.g. soil, biosolids or faecal slurry (with sterilised matrix equivalents as negative controls), incubated and then seeded onto media containing the study compound and a selective antibiotic to recover marked recipient strains demonstrating resistance. Plasmids or the entire genome of the recipient could then be cloned into small insert expression vectors, transformed into *E. coli*, or other hosts, and seeded

back onto media containing the study compound. In this way novel resistance determinants would be identified.

Alternatively functional metagenomics could be used to identify novel resistance determinants in metagenomic DNA (Allen et al. 2009). In brief, DNA would be extracted from an environmental sample, cloned into an expression vector and transformed into a bacterial host such as *E. coli*. Transformants can then be screened on the study compound and resistance genes identified using transposon mutagenesis followed by sequencing and bioinformatic analyses. This would allow detection of novel resistance determinants that may not be plasmid borne, yet may transfer to other pathogens.

Dose-response data needs – for Processes 3, 5, and 6

The authors were unaware of dose-response data representing a combined ARG and a recipient, previously susceptible pathogen dose and human or animal disease (processes 3 and 5, Figure 1). In contrast, as discussed in a section above, various examples illustrate increased morbidity and mortality when humans are exposed to pARB. Hence, existing published dose-response models for non-resistant pathogens (Haas et al. 1999) may not be appropriate to use beyond the endpoint of infection, and further dose-response models addressing people of various life-stages need to be described and summarized to facilitate pARB risk assessments. There is also a need to develop dose-response information for secondary illness endpoints (sequelae) resulting from pARB infections.

Regarding antibiotic concentration-time of exposure giving rise to selection of pARB within a human (for co-uptake of eARB and sensitive pathogen), safety could be based on the effective concentration for the specific antibiotic under consideration. In other words, screening values to

determine whether further action is warranted could be derived from the acute or mean daily antibiotic intake, with uncertainty factors applied as appropriate, until future research is undertaken on pathogen antibiotic-response changes in the presence of specific antibiotic treatment. Alternatively, epidemiological data from existing clones of antibiotic-resistant strains (e.g. NDM-1) could provide useful data for dose-response and exposure assessments.

Options for ranking risks – overall HHRA

In the absence of fully quantitative data to undertake a HHRA, risk-ranking approaches based on exposure assessment modelling could be adopted and developed to inform allocation of resources for data generation as part of a human health risk assessment of ARB. (Evers et al. 2008) present one such approach in the context of food safety for estimating the relative contribution of *Campylobacter* spp. sources and transmission routes on exposure per person-day in the Netherlands. Their study included 31 transmission routes, related to direct contact with animals, ingestion of food and water, and resulted in a ranking of the most significant sources of exposure. Although their study focused on foodborne transmission routes and did not deal with antibiotic resistant *Campylobacter* strains, a similar approach could be applied to estimate human exposure to ARB hazards considering the environmental exposure pathways described by Evers et al. (2008). This would require data on the prevalence of ARG, ARB as well as levels of antibiotics present in all exposure routes to be considered in the risk assessment. While such an approach is probably not currently feasible, improved environmental data for a select number of pathogen-gene combinations could be developed in the future.

An alternative approach to capturing expert and other stakeholder knowledge could be to develop a Bayesian network based on expert knowledge and add to that as data become available to improve the knowledge base, as described for campylobacters in foods by (Albert et al. 2008).

Conclusions

Given the intent of this review to be of value to an international audience, and that the precautionary approach is used in many jurisdictions, there are many risk management approaches that make sense to implement now, before antibiotic-resistance issues worsen, as indicated in the related risk management paper resulting from the workshop (Pruden et al. 2013). Furthermore, many current management schemes now start the whole process from a management perspective and drill down to more quantitative assessments only when needed to make a better management action, such as in the World Health Organization's Water Safety Plans (WHO 2009). In this paper we provide a proposal for the inclusion of environmental aspects of antibiotic resistance development in the processes of any HHRA addressing ARB. In general terms, MRA appears suitable to address environmental human health risks posed by the environmental release of antibiotics, ARB and ARG, yet there are too many data gaps to realize that goal today. Further development of such an approach requires data mining from previous epidemiological investigations to aid in model development, parameterization and validation, as well as the collection of new information, particularly related to conditions and rates of ARB development in various 'hot-spot' environments, and for various human health dose-response unknowns identified in this paper. In the near-term, options likely to provide a first-pass assessment of risks seem likely to be based on MCDA approaches, which could be facilitated by Bayesian Network models. Once some 'trust' in such MRA models has been achieved, they may

well facilitate scenario testing of what control points may be most effective in reducing risks and which risk-driving attributes should be specifically considered and minimised during the development of novel antibiotics.

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Figure Legend

Figure 1. Conceptual model describing the environmental pathways that result in an increased risk of human and animal infection with antibiotic resistant bacteria. The first six processes (*italics*) are further described in the text. The last two processes are not driven by environmental factors and not discussed in detail.

